GROWTH-PROMOTING EFFECTS OF HYDROLYZED NUCLEIC ACIDS, NUCLEOTIDES, AND NUCLEOSIDES ON ENDAMOEBA HISTOLYTICA

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Endamoeba histolytica has not yet been maintained indefinitely in pure culture, although bacteria-free cultures have been carried for periods of up to one month in growth factor-fortified media (Nakamura and Baker, 1956). Jacobs (1947) and Shaffer and Frye (1948) have also grown the amebas in media containing no or relatively few multiplying bacteria. Therefore, it becomes apparent that although bacteria contribute tremendously to the growth and multiplication of the amebas, they are not absolutely essential and that perhaps amebic growth can occur in a semi-synthetic medium if supplied the necessary growth-promoting factors. It has been shown that purines, pyrimidines, citrovorum factor, and ribose-5-phosphate can substitute partially for bacterial association and permit bacteria-free cultures of *E. histolytica* to multiply for a limited period. However, ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) tested singly or in combination failed to promote amebic growth; on the other hand, dialysates of media containing RNA and DNA preconditioned by bacterial growth contained ameba-stimulatory substances which permitted seven sub-cultures of the amebas in the absence of associated bacteria (Nakamura and Baker, 1956). It was postulated that bacterial action on the nucleic acids produced catabolic intermediate(s) which were essential to the nutrition of the amebas. In order to determine more exactly the specific components in the nucleic acid digest which were ameba-stimulatory, nucleic acids were hydrolyzed by enzymatic, acid, and alkaline hydrolysis; the dialysates of the hydrolysates were studied for their effects on *E. histolytica* under bacteria-free conditions. Furthermore, nucleosides and nucleotides, obtained from commercial sources, were also assayed for their activity on the growth of the amebas.

Materials and Methods

Organism and the assay medium

Strains of *E. histolytica* employed in these experiments consisted of: (1) NRS, obtained from Dr. Quentin M. Geiman, Stanford University School of Medicine, San Francisco, California, (2) HUS-100, isolated from the stool of a carrier during an outbreak of amebiasis in Indiana in 1953, obtained from Dr. Chia-Tung Pan, Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts, and (3) UC, also obtained from Dr. Pan. Stock cultures of the amebas, containing a mixed bacterial flora, were maintained in a modified Boeck-Drbohlav (1925) medium. The assay methods were essentially identical with those described earlier (Nakamura, 1955; Nakamura and Baker, 1956; Nakamura and

Jonsson, 1956). Coagulated egg slants were overlaid with a liquid phase consisting of glucose (0.5%), sodium thioglycollate (0.3%), penicillin G (10,000 units/ ml. final concentration), streptomycin (5000 units/ ml. final concentration), horse serum-Ringer solution (1/5), rice powder (approximately 10 mg.), and a vaspar (vaseline and paraffin, 1/1) seal. The volume of the overlay fluid was four ml. The dialysates and the nucleosides and nucleotides assayed were added to the liquid phase of the medium.

The inocula consisted of 2 drops of stock cultures adjusted to contain approximately 15–20 amebas per low power field. The bacteria introduced with the ameba inocula were sterilized within 4–6 hours by the combinations of antibiotics used. The culture tubes were incubated for 3–4 days at 37° C.; ameba counts were made by taking the sediment from each culture tube (in duplicate), placing a few drops on a clean slide, covering with a cover slide (22 × 22 mm.) and counting the number of amebas per low power field. Ten fields were counted and an average count recorded. At the same time the amebas were transferred to tubes containing identical nutritional components. Control tubes consisted of media lacking only the materials being assayed. Positive controls consisted of media fortified with the growth factors ribose-5-phosphate and adenosinetriphosphate.

Hydrolysis of nucleic acids

The method of Kerr *et al.* (1949) was used for the acid hydrolysis of RNA. Fifty mg. of RNA were placed in a test tube with 5 ml. of 2 N sulfuric acid. The tube was placed in boiling water for 30 minutes. After hydrolysis the contents of the tube were diluted to 25 ml. with water. Acid hydrolysis of DNA was accomplished by placing 50 mg. of DNA in 5 ml. N sulfuric acid and refluxing in boiling water for 2 hours. The hydrolysate was adjusted to pH 6.5 with alkali.

The method of Volkin and Carter (1951) was used for the alkaline hydrolysis of RNA. Fifty mg. of RNA, dissolved in 3 ml. of 0.5 N NaOH, were kept at 37° C. for 17 hours. The digest was diluted with water to 0.02 N NaOH and finally neutralized. DNA was hydrolyzed using a modified method of Marrian *et al.* (1951).

Enzymatic hydrolysis of RNA was accomplished by suspending 50 mg. of RNA in 5 ml. water and adjusting to pH 7.2 with dilute NaOH. Then 5 mg. of crystalline ribonuclease, dissolved in 1 ml. of 0.1 *M* phosphate buffer at pH 7.2, was added to the nucleic acid solution under stirring. As the reaction progressed the solution was maintained at pH 7.2 with the addition of 0.05 N NaOH. The temperature of the digest was maintained between 25–27° C. Hydrolysis was complete in two hours. The method of Smith and Markham (1952) was used for the enzymatic digestion of DNA; the DNA was digested with desoxyribonuclease (20 ugm./ml.) in 0.005 *M* magnesium sulfate at pH 7.0 for 18 hours. The digests were dialyzed in water and the dialysates tested for their growth-promoting activity.

Results

As is evident in Tables I, II, and III, enzyme-hydrolyzed nucleic acids (both RNA and DNA) yielded a product which was stimulatory to the growth of *E. histolytica*. In all of the experiments enzyme-hydrolyzed RNA consistently stimulated the amebas slightly more than the enzyme-hydrolyzed DNA preparation. Al-

TABLE I

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; strain NRS

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	15	1
Basal + enzyme-hydrolyzed RNA	4	79
Basal + enzyme-hydrolyzed DNA	4	55
Basal + alkaline-hydrolyzed RNA	4	47
Basal + alkaline-hydrolyzed DNA	4	50
Basal + acid-hydrolyzed RNA	4	0
Basal + acid-hydrolyzed DNA	4	0
Basal + unhydrolyzed RNA	8	10
Basal + unhydrolyzed DNA	8	7
Basal + adenosine (0.1 mg./ml.)	4	41
Basal + guanosine (0.1 mg./ml.)	4	49
Basal + thymidine (0.1 mg./ml.)	4	59
Basal + adenylic acid (0.1 mg./ml.)	4	70
Basal + guanylic acid (0.1 mg./ml.)	4	66
Basal + thymidylic acid (0.1 mg./ml.)	4	83
Basal + uridylic acid (0.1 mg./ml.)	4	47

TABLE II

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; strain HUS-100

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	15	0.4
Basal + enzyme-hydrolyzed RNA	4	64
Basal + enzyme-hydrolyzed DNA	4	40
Basal + alkaline-hydrolyzed RNA	4	51
Basal + alkaline-hydrolyzed DNA	4	54
Basal + acid-hydrolyzed DNA	4	1
Basal + acid-hydrolyzed RNA	4	0
Basal + unhydrolyzed RNA	4	0
Basal + unhydrolyzed DNA	4	1
Basal + adenosine (0.1 mg./ml.)	4	39
Basal + guanosine (0.1 mg./ml.)	4	34
Basal + thymidine (0.1 mg./ml.)	4	43
Basal + adenylic acid (0.1 mg./ml.)	4	57
Basal + guanylic acid (0.1 mg./ml.)	4	68
Basal + thymidylic acid (0.1 mg./ml.)	4	49
Basal + uridylic acid (0.1 mg./ml.)	4	33

kaline hydrolysates of nucleic acids were also stimulatory to the amebas. However, acid-hydrolyzed nucleic acids were without ameba-stimulatory properties in studies on all three strains of *E. histolytica*. Unhydrolyzed nucleic acids were inactive, except for a slight effect on the NRS strain, as was to be expected according to the earlier data of Nakamura and Baker (1956). The nucleosides adenosine, guanosine, and thymidine stimulated the HUS-100 and NRS strains but not the UC strain. The nucleotides adenylic acid, guanylic acid, thymidylic acid, and uridylic acid were active as growth factors for all three strains of amebas tested, although

TABLE III

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; UC strain

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	18	2
Basal + enzyme-hydrolyzed RNA	4	83
Basal + enzyme-hydrolyzed DNA	4	69
Basal + alkaline-hydrolyzed RNA	4	40
Basal + alkaline-hydrolyzed DNA	4	40
Basal + acid-hydrolyzed RNA	4	3
Basal + acid-hydrolyzed DNA	4	2
Basal + unhydrolyzed RNA	4	1
Basal + unhydrolyzed DNA	4	0
Basal + adenosine (0.1 mg./ml.)	4	0
Basal + guanosine (0.1 mg./ml.)	4	2
Basal + thymidine (0.1 mg./ml.)	4	5
Basal + adenylic acid (0.1 mg./ml.)	4	61
Basal + guanylic acid (0.1 mg./ml.)	4	68
Basal + thymidylic acid (0.1 mg./ml.)	4	90
Basal + uridylic acid (0.1 mg./ml.)	4	56

the degree of activity as growth-promoting factors varied slightly from compound to compound.

Attempts to maintain bacteria-free subcultures on media containing hydrolyzed nucleic acids, nucleotides, or nucleosides were generally unsuccessful. The longest culture maintained on enzyme-hydrolyzed RNA and enzyme-hydrolyzed DNA was 5 transfers for a total of approximately 15 days. Sterility tests indicated the absence of viable bacterial cells. In media containing alkaline-hydrolyzed nucleic acids, two to three subcultures were usually possible; however, the total amebic populations were considerably lower than in the enzyme-treated nucleic acid media. Only one subculture with a meager ameba count was possible in the experiments containing nucleosides and nucleotides as growth factors.

DISCUSSION

The data in this report are in agreement with earlier reports that nucleic acids preconditioned by bacterial growth produce some catabolic metabolite(s) which are essential for amebic growth in the absence of living bacteria. In these experiments, enzymatic and alkaline digestion, rather than bacterial preconditioning, yielded ameba-growth-promoting factors. It is indeed difficult to explain the absence of similar stimulatory activity in the acid-hydrolyzed nucleic acid solutions. In studies with *Trichomonas vaginalis,* Sprince *et al.* (1953) reported that acid hydrolysis of RNA destroyed the growth-promoting effect of RNA whereas alkaline hydrolysis of RNA left intact this growth-promoting factor. They also reported that acid, alkaline, and enzymatic hydrolysis of DNA destroyed the growth-promoting effects of DNA. These results, however, are not quite analogous to the data in this paper since Sprince *et al.* (1953) were dealing with DNA and RNA which were established as growth-promoting factors for *Trichomonas;* in the case of *Endamoeba histolytica,* DNA and RNA in themselves do not stimulate amebic growth.

It is highly probable that the ameba-growth-stimulatory action of nucleic acid hydrolysates was not due solely to the nucleosides and nucleotides formed during the digestion. Smith and Markham (1952) have found that enzyme digestion of DNA produces many dinucleoside monophosphates; Markham and Smith (1952) reported that products of enzyme hydrolysis of RNA were largely cyclic pyrimidine nucleotides and only traces of adenylic and guanylic acids were found. On the other hand, alkaline digestion of RNA produces guanylic, adenylic, and uridylic acids (Magasanik and Chargaff, 1951).

The growth factor effects of purified nucleosides and nucleotides indicate the importance of these substances in amebic nutrition; these substances play a role in the synthesis of nucleic acids and pyridine nucleotides. Diphosphopyridine nucleotide has been shown to be necessary for amebic growth in the absence of bacteria (Nakamura and Baker, 1956). Johnson (1953) similarly showed that cytidylic and guanylic acids were growth factors for *Paramecium multimicronucleatum*.

There is evidence that different strains of *E. histolytica* possess different growth factor requirements. The nucleosides, which were highly active for the NRS and HUS-100 strains, were without activity on the UC strain. It is possible that the UC strain can synthesize its own nucleoside but that it cannot phosphorylate the nucleoside into the nucleotide which it apparently requires. In the cases of the NRS and HUS-100 strains, it appears logical to assume that they can synthesize neither nucleosides nor nucleotides, yet when supplied these two growth factors exogenously, the amebas can synthesize their own nucleic acids. A strong point in favor of this assumption is the fact that pre-formed nucleic acids do not aid amebic growth appreciably whereas the nucleosides and nucleotides are highly stimulatory.

Summary

1. Enzyme and alkaline hydrolysates of ribonucleic and desoxyribonucleic acids contained growth-promoting factors for *Endamoeba histolytica*. Acid hydrolysates of nucleic acids, however, were without this stimulatory activity on the amebas.

2. Nucleosides, adenosine, guanosine, and thymidine, were stimulatory to the NRS and HUS-100 strains but not for the UC strain. Nucleotides, adenylic acid, guanylic acid, thymidylic acid, and uridylic acid, were highly stimulatory for the growth of all three strains of *E. histolytica* studied.

LITERATURE CITED

Boeck, W. C., and J. Drbohlav, 1925. The cultivation of *Endamoeba histolytica*. *Amer. J. Hyg.*, **5**: 371–407.

Jacobs, L., 1947. The elimination of viable bacteria from cultures of *Endamoeba histolytica* and the subsequent maintenance of such cultures. *Amer. J. Hyg.*, **46**: 172–176.

Johnson, W. H., 1953. Discussion of papers on ciliate nutrition. *Ann. N. Y. Acad. Sci.*, **56**: 969–971.

Kerr, S. E., K. Seraidarian and M. Wargon, 1949. Studies on ribonucleic acid. II. Methods of analysis. *J. Biol. Chem.*, **181**: 761–771.

Magasanik, B., and E. Chargaff, 1951. Studies on the structure of ribonucleic acids. *Biochim. et Biophys. Acta*, **7**: 396–412.

Markham, R., and J. D. Smith, 1952. The structure of ribonucleic acids. I. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. *Biochem. J.*, **52**: 552–557.

Marrian, D. H., V. L. Spicer, M. E. Balis and G. B. Brown, 1951. Purine incorporation into pentose nucleotides of the rat. *J. Biol. Chem.*, **189**: 533–541.

Nakamura, M., 1955. Growth factors for *Endamoeba histolytica*. *Proc. Soc. Exp. Biol. Med.*, **89**: 680–682.

Nakamura, M., and E. E. Baker, 1956. Nutritional requirements of *Endamoeba histolytica*. *Amer. J. Hyg.*, **64**: 12–22.

Nakamura, M., and S. Jonsson, 1956. The effect of antimetabolites on the growth of *Endamoeba histolytica*. I. Purine and pyrimidine analogs. *Arch. Biochem. and Biophys.*, **66**: 183–189.

Shaffer, J. G., and W. W. Frye, 1948. Studies on the growth requirements of *Endamoeba histolytica*. I. Maintenance of a strain of *E. histolytica* through one hundred transplants in the absence of an actively multiplying bacterial flora. *Amer. J. Hyg.*, **47**: 214–221.

Smith, J. D., and R. Markham, 1952. The enzymatic breakdown of deoxyribosenucleic acids. *Biochim. et Biophys. Acta*, **8**: 350–351.

Sprince, H., R. Goldberg, G. Kucker and R. S. Lowy, 1953. The effect of ribonucleic acid and its nitrogenous constituents on the growth of *Trichomonas vaginalis*. *Ann. N. Y. Acad. Sci.*, **56**: 1016–1027.

Volkin, E., and C. E. Carter, 1951. The preparation and properties of mammalian ribonucleic acids. *J. Amer. Chem. Soc.*, **72**: 1516–1519.